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(71) Applicant (for all designated States except US): **NORTH-WESTERN UNIVERSITY** [US/US]; 2225 North Campus Drive, Evanston, IL 60208 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **STUPP, Samuel, I.** [US/US]; 57 E. Delaware Avenue, Apartment 2802, Chicago, IL 60611 (US). **HARTGERINK, Jeffrey, D.**

[US/US]; 1115 Elmwood Avenue, Evanston, IL 60202 (US). **BENIASH, Elia** [US/US]; 4615 Davis Street, Skokie, IL 60076 (US).

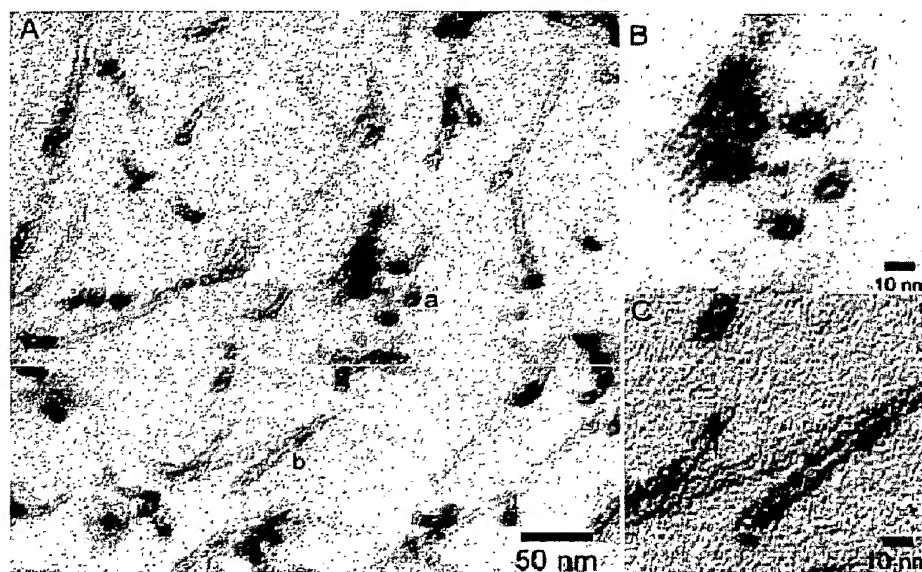
(74) Agent: **MILLER, Raymond, A.**; Pepper Hamilton LLP, 50th Floor, 500 Grant Street, Pittsburgh, PA 15219 (US).

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[Continued on next page]

(54) Title: PEPTIDE AMPHIPHILE SOLUTIONS AND SELF ASSEMBLED PEPTIDE NANOFIBER NETWORKS



(57) Abstract: Peptide amphiphile self assembly and gelation to form nanofiber networks having cells within the network are described. The molecular structure of peptide amphiphiles and compositions including them suitable for forming nanofiber networks with cells under physiological conditions are also described. Methods to incorporate dissociated cells into self-assembled peptide amphiphile gels for molding of implants, in situ molding in animals, and injection of peptide amphiphile and cell compositions into an animal for tissue engineering and tissue repair applications are disclosed. The methods and compositions of the present invention are used to grow animal cells in a self assembled nanofiber network.



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## **PEPTIDE AMPHIPHILE SOLUTIONS AND SELF ASSEMBLED PEPTIDE NANOFIBER NETWORKS**

### **CROSS REFERENCES TO RELATED APPLICATIONS**

**[0001]** This application claims priority from U.S. Provisional Application Serial Number 60/369,638 filed April 2, 2002, the contents of which are included herein by reference in their entirety.

### **GOVERNMENT INTERESTS**

**[0002]** The United States Government may have certain rights to this invention pursuant to work funded thereby at Northwestern University under grants from the Department of Energy Grant No. DE-FG02-00ER45810, Office of Naval Research Grant No. N00014-99-1-02396, and the National Science Foundation Grant No. DMR-9996253.

### **BACKGROUND**

**[0003]** Self-assembly is a mechanism used in biological systems to make complex membranes, structural materials, and tissues by organizing starting materials such as dissolved minerals, proteins, and cells. Bone and shell are examples of complex biological composite materials which are assemblies of mineral crystallites like calcite or hydroxyapatite within a scaffold of a protein such as collagen. Tissues are organized assemblies of cells. While a detailed understanding of the mechanism of how individual cells interact to form a tissue is not well understood, it has been observed that cells within a matrix, for example Madin-Darby Canine Kidney cells embedded in a collagen type I matrix, will form a tissue like structures called cysts from the cells.

**[0004]** Techniques of tissue engineering employing biocompatible scaffolds or matrices to organize cells provide viable alternatives to prosthetic materials currently used in

prosthetic and reconstructive surgery (e.g. craniomaxillofacial surgery). These materials also hold promise in the formation of tissue or organ equivalents to replaced diseased, defective, or injured tissues. In addition to their use in the biocompatible scaffolds, biodegradable materials may be used for controlled release of therapeutic materials (e.g. genetic material, cells, hormones, drugs, or pro-drugs) into a predetermined area of an animal. However, polymers used to create these scaffolds, such as polylactic acid, polyorthoesters, and polyanhydrides, are generally difficult to mold and are hydrophobic, resulting in, among other things, poor cell attachment. Moreover, manipulations of the polymers must be performed prior to implantation of the polymeric material into an animal.

**[0005]** It would be desirable to have a scaffold or matrix for culturing cells and for tissue grafting that is suitable for implantation into an animal. It would be desirable that the matrix be easy to mold into a variety of shapes, that it be easily wet by fluids in the animal, and that it is easy to distribute cells throughout the matrix prior to implantation. The matrix should be biologically compatible, and should enhance the attachment, growth, and division of cells within the scaffold. It would be highly desirable that such a scaffold be made under physiological conditions enabling scaffolds to be made within the animal.

## SUMMARY

**[0006]** An embodiment of the present invention is directed to compositions and methods of making a scaffold comprised of peptide amphiphiles and cells. The scaffold, which is gel or fibrous network or matrix composed of self assembled peptide amphiphiles, may be made by combining a composition including peptide amphiphiles with a composition including cells to form a mixture. In the presence of polyvalent ions the peptide amphiphiles in the mixture form a gel or nanofiber network with cells included within the structure of the gel or fibrous network. The mixture of the peptide amphiphiles and cells may be injected

into an animal to form a gel including the cells within the animal. Alternatively the mixture of peptide amphiphiles and cells may be poured or injected into a mold thereby forming a self-assembled peptide amphiphile gel including the cells in the shape of the mold. The molded item may be used as an implant in an animal for growing cells and tissues

**[0007]** Gelation of the peptide amphiphile solution may be initiated prior to implantation in the animal or gelation of the peptide amphiphile solution may be initiated after the cell and peptide amphiphile solution is injected into the animal. Gelation of the peptide amphiphile solution occurs by using ions, altering the pH or changing the temperature of the peptide amphiphile and cell mixture.

**[0008]** Cells which may be incorporated into the peptide amphiphile scaffold include but are not limited to chondrocytes, muscle cells, fibroblasts, and cells acting primarily to synthesize, secrete or metabolize materials; pluripotent cells, stem cells, precursor cells and combinations thereof; and myocytes, adipocytes, fibrocytes, ectodermal cell, muscle cells, osteoblast, chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.

**[0009]** One embodiment of the present invention is a kit for formation of tissue at a site in a patient. The kit may comprise individual components of the mixture to form a gel with cells, such as peptide amphiphile, cells, and polyvalent ion, or it may comprise an injectable mixture of peptide-amphiphiles in solution with cells. The kit also includes means for injecting the cell and peptide amphiphile solution into the site in the patient where the cells are needed, or to where a tissue or gel including cells may be formed.

**[0010]** Another embodiment of the present invention is a composition including cells and peptide amphiphile, wherein cells and peptide amphiphiles are in solution, and the composition is capable of forming a gel upon exposure to physiological conditions.

Preferably some of the peptide component of said peptide amphiphile includes a residue with a functional moiety capable of intermolecular covalent bond formation such as a cysteine amino acid. Cells in the composition may include those such as pluripotent cells, stem cells, precursor cells and combinations thereof; myocytes, adipocytes, fibromyoblasts, ectodermal cell, muscle cells, osteoblast, chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.

[0011] In yet another embodiment of the present invention is composition comprising an aqueous solution of a peptide-amphiphile composition, a cellular component, and a reagent to induce gelation of said amphiphile composition.

[0012] In yet another embodiment of the present invention is a composition which includes a self assembled peptide-amphiphile gel and cells.

## DESCRIPTION OF THE DRAWINGS

[0013] In part, other aspects, features, benefits and advantages of the embodiments of the present invention will be apparent with regard to the following description, appended claims and accompanying drawings where:

[0014] Figure 1A illustrates chemical structure of a peptide-amphiphile (Molecule 3 ( $C_{15}H_{31}C(O)$ -SEQ ID NO:2-SEQ ID NO:5)) that may be considered a platform for preferred embodiments of the present application; Figure 1A illustrates chemical connectivity of a peptide-amphiphile indicating five important regions for consideration in the design of the molecule; Figure 1B illustrates via space filling model of the same molecule; Figure 1C schematically illustrates the self-assembly of the individual peptide amphiphiles into a nanofiber;

[0015] Figure 2 corresponds to the chemical structures of Molecule 1 and Molecule 2 described and utilized in a preferred embodiment of the present invention;

[0016] Figure 3 is an optical micrograph of mouse calvaria cells embedded in the peptide-amphiphile gel according to an embodiment of the present invention;

[0017] Figure 4 illustrates TEM micrograph of mouse calvaria cell surrounded by nanofibers.

[0018] Figure 5 illustrates TEM micrograph of the nanofiber network surrounding the cells.

### DETAILED DESCRIPTION

[0019] Before the present compositions and methods are described, it is to be understood that this invention is not limited to the particular molecules, compositions, methodologies or protocols described, as these may vary. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

[0020] It must also be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to a “cell” is a reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated by reference. Nothing herein is to be

construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0021] The present invention is directed to various modes of self-assembly and controlled self-assembly of peptide-amphiphiles. The self-assembly of peptide amphiphiles into cylindrical fibrils has been described ( J.D. Hartgerink, E. Beniash and S.I. Stupp, Science 294, 1683-1688, 2001; and ). More particularly, preferred embodiments of the present invention are directed to methods of J.D. Hartgerink, E. Beniash and S.I. Stupp, PNAS, 99, 5133-5138, 2002). More particularly, preferred embodiments of the present invention are directed to methods and compositions for embedding living cells into a self-assembled peptide-amphiphile nanofiber network at physiological conditions. The formation of a self-supporting matrix or solid comprised of these nanofibers under physiological conditions affords the opportunity to utilize these materials alone or in conjunction with embedded cells for a wide range of purposes, including many in situ applications. While not wishing to be bound by theory, it appears that the availability, or addition of, polyvalent metal in cell ions in cell culture or under physiological conditions induces formation of a nanofiber network at physiological conditions. Depending on the particular peptide amphiphile, or mixture of peptide amphiphiles utilized, other conditions may be used to induce self assembly of the nanofibers. These conditions include ions, pH, cell culture medium oxidizing conditions, and physiological conditions.

[0022] The peptide-amphiphile (PA) compositions of the present invention can be synthesized using preparatory techniques well-known to those skilled in the art - preferably by standard solid phase chemistry, with alkylation of the N-terminus of the peptide. Mono or di-alkyl moieties attached to the N or C termini of peptides have been reported to influence their aggregation and secondary structure in water in both synthetic and natural systems. (J. S. Martinez et al. Science 287, 1245-1247 (2000); K Yamada et al. Chem. Lett 10 1713-1716



(1984); Yu et al. *Methods Enzymol.* 289, 571-587 (1997); KC Lee et al. *Langmuir* 15, 5500-5508 (1999). According to existing knowledge of amphiphile self-assembly, an alkyl tail with 16 carbon atoms (Figure 1A, region 1) coupled to an ionic peptide should create an amphiphile that assembles in water into cylindrical micelles because of the amphiphiles overall conical shape. The alkyl tails of the peptide amphiphile pack in the center of the micelle with the peptide segments exposed to an aqueous environment as shown in Figure 1C. While the present invention has been described with respect to alkyl tails, the hydrophobic nature and function of the tail may also be effected by tails including various degrees of unsaturation through alkene and alkyne groups in the tail as would be known and could readily be made by those skilled in the art. These cylindrical micelles can be viewed as fibers in which the chemistry of the peptide region is repetitively displayed on their surface. Similar peptide amphiphile molecules can also be designed to provide micelles having structural shapes that may differ from a fiber like appearance. Other compositions may also be used to induce predetermined geometric orientations of the self-assembled amphiphile peptides.

[0023] Figure 1A illustrates chemical connectivity of a peptide-amphiphile indicating five important regions for consideration in the design of the molecule. Region 1 is a simple hydrophobic tail, which is preferably an alkyl tail, that can be a variety of sizes but must be greater than 6 carbon atoms in length. Region 2 can be used for cross-linking if cysteine amino acids are selected as shown in Figure 1A. If cross-linking is not desired other amino acids such as alanine, see Molecule 1 and Molecule 2 in Figure 2, may be used in this region. Region 3 is a flexible linker between the cross-linking region and the hydrophilic head group and may be composed of glycine or other flexible amino acids. Regions 4 & 5 compose the hydrophilic head group and may be composed of essentially any hydrophilic amino acids such as phosphorylated serine, glutamic acid, lysine, etc. and can be used to create cell

signaling sequences such as RGD (SEQ ID NO: 5), IKVAV (SEQ ID NO:6), YIGSR (SEQ ID NO:7) and others. Figure 1B illustrates via space filling model, the same molecule shown in Figure 1A; Figure 1C schematically illustrates the self-assembly of the individual peptide-amphiphiles into a nanofiber.

**[0024]** Various other peptide amphiphile compositions of this invention and conservatively modified variants of the peptide sequences, SEQ ID NO:1-7 may be prepared in analogous fashion, as would be known to those skilled in the art and aware thereof, using known procedures and synthetic techniques or straight-forward modifications thereof depending upon a desired amphiphile composition or peptide sequence. For example, modifications of SEQ ID NO: 1-3 may include but are not limited to those in which one or more of the cysteine, alanine, glycine, glutamic acid, or serine amino acids has been deleted, substituted for by another amino acid, or phosphorylated. Hydrophilic head groups such as (SEQ ID NO:5-7) and conservative modifications thereof may include modifications such as the addition or removal of amino acids or use of other peptide sequences for interacting with cells. Peptide amphiphiles may be in their fully protonated form, partially protonated form, or as acid addition salts.

**[0025]** Biocompatible and or biodegradable gels are useful for delivering isolated cells into a patient to create an organ equivalent or tissue such as cartilage. The gels promote engraftment and provide three-dimensional templates for new cell growth. The resulting tissue may be generally similar in composition and histology to naturally occurring tissue.

**[0026]** In one embodiment of the present invention, cells are suspended in a self-assembling peptide-amphiphile solution and injected directly into a site in a patient, where the self-assembled peptide amphiphiles gel organizes into a matrix having cells dispersed therein.

[0027] In a second embodiment, cells are suspended in a self-assembled peptide amphiphile gel which is then poured or injected into a mold having a desired anatomical shape. The gel then organizes to form a matrix having cells dispersed therein which can be implanted into a patient or animal. Preferably, the self-assembled peptide amphiphile gel degrades, leaving only the resulting tissue.

[0028] Peptide components of the invention preferably include naturally occurring amino acids. However, incorporation of artificial amino acids such as beta or gamma amino acids and those containing non-natural side chains, and/or other similar monomers such as hydroxyacids are also contemplated, with the effect that the corresponding component is peptide-like in this respect. One example already tested includes an amino acid substituted with a thiophene moiety so that polymerization can produce electrically conductive and/or fluorescent materials.

[0029] Various aspects of the present invention can be described with reference to the peptide amphiphile as it is generally illustrated in Figure 1A. Consistent with broader aspects of this invention, other compositions can be prepared in accordance with the invention and used for the self-assembly of micelles.

[0030] Cellular adhesion or interaction between the cells and the nanofibers in the gel or matrix may be promoted by a particular sequence of the peptide component. In particular, the IKVAV sequence (SEQ ID NO:6) has been identified in other context as important for neuron growth and development. Accordingly, the amphiphile compositions of the present invention can include a peptide component having a sequence for corresponding use. Mixtures of peptide amphiphiles may be utilized to achieve a desired cell engraftment and cell division within the gel. Preferably the biological signals present on the surface of the nanofibers in the gel are similar in number and density to those found in nature. It should be noted that the peptide-amphiphile compositions do not require cysteine residues: while such a

peptide sequence can be used to enhance intermolecular nanofiber stability, it is not required form micelle formation in the first instance. The number of cysteine amino acids in the peptide as well as their location within the peptide may be varied as would be known to those skilled in the art. In the instance where cysteine residues are utilized it would be preferable to utilize biocompatible reducing agents known in the art to control disulfide formation at physiological conditions.

[0031] Cells can be obtained directly from a donor, from a culture of cells from a donor, or from established cell culture lines. In the preferred embodiments, cells are obtained directly from a donor, washed and implanted directly in combination with the peptide-amphiphile solution. The cells are cultured using techniques known to those skilled in the art of tissue culture.

[0032] Cells to be implanted may be dissociated using standard techniques such as digestion with a collagenase, trypsin or other protease solution. Preferred cell types are mesenchymal cells, especially smooth or skeletal muscle cells, myocytes (muscle stem cells), fibroblasts, chondrocytes, adipocytes, fibromyoblasts, and ectodermal cells, including ductile and skin cells, hepatocytes, Islet cells, cells present in the intestine, and other parenchymal cells, osteoblasts and other cells forming bone or cartilage. In some cases it may also be desirable to include nerve cells. Cells can be normal or genetically engineered to provide additional or normal function. Methods for genetically engineering cells with retroviral vectors, polyethylene glycol, or other methods known to those skilled in the art can be used.

[0033] Cells are preferably autologous cells, obtained by biopsy and expanded in culture, although cells from close relatives or other donors of the same species may be used with appropriate immunosuppression. Immunologically inert cells, such as embryonic or fetal cells, stem cells, and cells genetically engineered to avoid the need for immunosuppression can also be used. Methods and drugs for immunosuppression are known

to those skilled in the art of transplantation. A preferred compound is cyclosporin using the recommended dosages.

[0034] In the preferred embodiment, cells are obtained by biopsy and expanded in culture for subsequent implantation. Cells can be easily obtained through a biopsy anywhere in the body of an animal. For example, skeletal muscle biopsies can be obtained easily from the arm, forearm, or lower extremities, and smooth muscle can be obtained from the area adjacent to the subcutaneous tissue throughout the body. To obtain either type of muscle, the area to be biopsied can be locally anesthetized with a small amount of lidocaine injected subcutaneously. Alternatively, a small patch of lidocaine jelly can be applied over the area to be biopsied and left in place for a period of 5 to 20 minutes, prior to obtaining biopsy specimen. The biopsy can be effortlessly obtained with the use of a biopsy needle, a rapid action needle that makes the procedure extremely simple and almost painless. With the addition of the anesthetic agent, the procedure would be entirely painless. This small biopsy core of either skeletal or smooth muscle can then be transferred to media consisting of phosphate buffered saline. The biopsy specimen is then transferred to the lab where the muscle can be grown utilizing the explant technique, wherein the muscle is divided into very small pieces which are adhered to culture plate, and serum containing media is added. Alternatively, the muscle biopsy can be enzymatically digested with agents such as trypsin and the cells dispersed in a culture plate with any of the routinely used medias. After cell expansion within the culture plate, the cells can be easily passaged utilizing the usual technique until an adequate number of cells are achieved.

[0035] Cell attachment and viability of implanted cells may be assessed using scanning electron microscopy, histology, and quantitative assessment with radioisotopes. The function of the implanted cells may be determined using a combination of the above-techniques and functional assays. For example, in the case of hepatocytes, placing a cannula

into the recipient's common bile duct can perform in vivo liver function studies. Bile can then be collected in increments. Bile pigments can be analyzed by high pressure liquid chromatography looking for underivatized tetrapyrroles or by thin layer chromatography after being converted to azodipyrroles by reaction with diazotized azodipyrroles ethylanthranilate either with or without treatment with P-glucuronidase. Simple liver function tests can also be done on blood samples, such as albumin production. Analogous organ function studies can be conducted using techniques known to those skilled in the art, as required to determine the extent of cell function after implantation. For example, islet cells of the pancreas may be delivered in a similar fashion to that specifically used to implant hepatocytes, to achieve glucose regulation by appropriate secretion of insulin to cure diabetes.

[0036] The techniques described herein can be used to provide multiple cell types, including genetically altered cells, within a three-dimensional scaffolding for the efficient transfer of large number of cells and the promotion of transplant engraftment for the purpose of creating a new tissue or tissue equivalent. It can also be used for immunoprotection of cell transplants while a new tissue or tissue equivalent is growing by excluding the host immune system.

[0037] Specific examples of cells that can be implanted as described herein include chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells. As used herein, "organ cells" includes hepatocytes, islet cells, cells of intestinal origin, cells derived from the kidney, and other cells acting primarily to synthesize and secrete, or to metabolize materials.

[0038] Biologically active materials may be added to the peptide amphiphile gel. The self-assembled peptide amphiphile matrix can be combined with humoral factors to promote cell transplantation and engraftment. For example, the self-assembled matrix can be combined with angiogenic factors, antibiotics, antiinflammatories, growth factors,

compounds which induce differentiation, and other factors which are known to those skilled in the art of cell culture.

[0039] For example, humoral factors could be mixed in a slow-release form with the cell-alginate suspension prior to formation of implant or transplantation. Alternatively, the self-assembled peptide amphiphile gel could be modified to bind humoral factors or signal recognition sequences prior to combination with isolated cell suspension.

[0040] The techniques described herein may be used for delivery of many different cell types to achieve different tissue structures. In the preferred embodiment, the cells are mixed with solutions of the self-assembling peptide amphiphile molecules and injected directly into a site where it is desired to implant the cells, prior to assembly of the self-assembled peptide amphiphile nanofibers. However, the matrix may also be molded and implanted in one or more different areas of the body to suit a particular application. This application is particularly relevant where a specific structural design is desired or where the area into which the cells are to be implanted lacks specific structure or support to facilitate growth and proliferation of the cells. The site, or sites, where cells are to be implanted may be determined based on individual need, as is the requisite number of cells. For cells having organ function, for example, hepatocytes or islet cells, the mixture of peptide amphiphiles and cells may be injected into the mesentery, subcutaneous tissue, retroperitoneum, properitoneal space, and intramuscular space. For formation of cartilage, the cells and peptide amphiphile mixture are injected into the site where cartilage formation is desired. One could also apply an external mold to shape the injected solution of cells and peptide amphiphile. Additionally, by controlling the rate of gelation, it is possible to mold the cell-self-assembled peptide amphiphile gel injected implant like one would mold clay. Alternatively, the mixture can be injected into a mold, the self-assembled peptide amphiphile gel allowed to self-assemble, and then implanting the molded gel structure.

**[0041]** Self-assembly and/or gelation under physiological conditions raises numerous implication regarding the end-use application and effect. A peptide-amphiphile mixture makes available a sol-gel system for the formation of micellular nanofibers in an aqueous environment at neutral and/or physiological pH conditions. Such a combination can be used to assemble nanofibers with a range of residues providing a variety of chemical or biological signals for corresponding cell adhesion, yielding enhanced properties with respect to tissue engineering or regenerative applications. It is contemplated that, alone or in conjunction with the other factors discussed herein, that preferred medical or therapeutic embodiments of such a system can be utilized.

**[0042]** In preferred embodiments, irrespective of any such factor, the peptide amphiphile composition(s) of such a system includes a peptide component having residues capable of intermolecular cross-linking. The thiol moieties of cysteine residues can be used for intermolecular disulfide bond formation through introduction of a suitable oxidizing agent or under physiological conditions. Conversely such bonds can be cleaved by a reducing agent introduced into the system or under reducing conditions. The concentration of cysteine residues can also be varied to control the chemical and/or biological stability of the nanofibrous system and therefore control the rate of therapeutic delivery or release of cells or other beneficial agent, using the nanofibers as the carriers. Furthermore, enzymes could be incorporated in the nanofibers to control biodegradation rate through hydrolysis of the disulfide bonds. Such degradation and/or the concentration of the cysteine residues can be utilized in a variety of tissue engineering contexts.

**[0043]** Embodiments of the present invention may be used for a variety of purposes. For example, custom-molded cell implants can be used to reconstruct three dimensional tissue defects, e.g., molds of human ears could be created and a chondrocyte-self-assembled peptide amphiphile gel replica could be fashioned and implanted to reconstruct a missing ear.



Cells can also be transplanted in the form of a three-dimensional structure that could be delivered via injection.

[0044] Different modes of self-assembly of the peptide-amphiphile molecules into cylindrical fibrils and other shapes have been described herein. This self-assembly generally occurs at predetermined concentrations (e.g. above 2.5 mg/ml) form self-supporting gel. In particular it has been found that addition of polyvalent metal ions induces gel formation of the negatively charged peptide-amphiphiles at physiological conditions. A number of negatively charged peptide-amphiphiles self-assembled into nanofibers by addition of polyvalent metal ions such as  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Cd}^{+2}$ ,  $\text{Fe}^{+2}$ ,  $\text{Gd}^{+3}$ .

### EXAMPLE 1

[0045] The present invention will be further understood by reference to the following non-limiting example. Figure 3 illustrates an optical micrograph of mouse calvaria cells embedded in a peptide amphiphile gel at the 5th day of the following experiment. The mouse calvaria cell line MC3T3-E1 was obtained from the University of Michigan, Ann Harbor, MI. Cells were cultured in a-MEM medium (Invitrogen), with 10% of fetal bovine serum (Hyclone) and 1% streptomycin/penicillin mixture (Invotrogen) at 37°C and  $\text{CO}_2$  concentration of 5%.

[0046] A number of negatively charged peptide-amphiphile compounds used in the study are listed below:

Table 1

Molecule	Formula	Charge
1	$\text{C}_{15}\text{H}_{31}\text{C}(\text{O})\text{-AAAAGGGS(P)KGE}$	-2
2	$\text{C}_{15}\text{H}_{31}\text{C}(\text{O})\text{-AAAAGGGS(P)RGD}$	-2
3	$\text{C}_{15}\text{H}_{31}\text{C}(\text{O})\text{-CCCCGGGS(P)RGD}$	-3

4	$C_{15}H_{31}C(O)-CCCCGGGEIKVAV$	-3
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[0047] It is well established that the RGD (SEQ ID NO:5) is a cell adhesion sequence found in almost all tissues. Therefore Molecule 2, ( $C_{15}H_{31}C(O)$ -SEQ ID NO:1-SEQ ID NO:5), as shown in Figure 2 and Table 1 was used in order to make a better contact between the cells and the nanofiber network. Molecule 1, ( $C_{15}H_{31}C(O)$ -SEQ ID NO:1-SEQ ID NO:4) has the same charge as Molecule 2, but does not contain the RGD (SEQ ID NO:5) signaling sequence. Molecule 1 was used as a filler in order to dilute RGD (SEQ ID NO:5) signal to a physiological level. This approach of mixing peptide-amphiphile molecules with different peptide sequences may be generally used for incorporation of different signaling sequences into one supramolecular nanofiber, in order to tailor the surface chemistry of the nanofibers to better reflect the chemistry of natural extracellular matrices that usually offer multiple signals to the cells. However, it may be desirable and is acceptable to use a single peptide amphiphile in accordance with the present invention. Tetra-alanine was incorporated into the molecules instead of tetra-cysteine since it does not require the presence of anti-oxidation agents. However it has also been shown that cysteine containing peptide-amphiphiles, such as Molecule 3 ( $C_{15}H_{31}C(O)$ -SEQ ID NO:2-SEQ ID NO:5) and Molecule 4 ( $C_{15}H_{31}C(O)$ -SEQ ID NO:3-SEQ ID NO:6), can be similarly gelled by cell culture media and divalent ions. In general any negatively charged peptide-amphiphile containing carboxylic acid and/or phosphate functionalities should function in a fashion similar to Molecules 1 ( $C_{15}H_{31}C(O)$ -SEQ ID NO:1-SEQ ID NO:4) and Molecule 2 ( $C_{15}H_{31}C(O)$ -SEQ ID NO:1-SEQ ID NO:5).

[0048] Mouse calvaria cell line MC3T3-E1, obtained from Dr. Lonnie Shea (University of Michigan, Ann Harbor, MI), were used in the study. Cells were cultured in the  $\alpha$ -MEM medium (Invitrogen), with 10% of fetal bovine serum (Hyclone) and 1% streptomycin/penicillin mixture (Invitrogen), at 37 °C and CO<sub>2</sub> concentration 5%.

[0049] 10 mg/ml aqueous solutions of molecules 1 and 2 (pH 7.5) were mixed in proportion 99:1, respectively. Cells were re-suspended in the medium, at density 6000 cells per  $\text{cm}^3$ . 200  $\mu\text{l}$  aliquots of the peptide-amphiphile solution were added to the wells of the 8 wells chamber slide (Fisher). 200  $\mu\text{l}$  aliquots of the cell suspension were added to the wells, and mixed with the peptide-amphiphile solution. Alternatively,  $\text{CaCl}_2$  was added to the cell suspension prior to mixing with the peptide-amphiphile solution. In this case final concentration of  $\text{Ca}^{+2}$  in the mixture was 25 mM, which is ~10 times higher than in the medium. In both cases gel formed instantly upon mixing of the cell suspension with the peptide-amphiphile solution. The samples were kept in an incubator at 37°C for 30 minutes in order to let the gel to mature. Then 500  $\mu\text{l}$  of the medium was added on the top of the formed gel. The samples were kept in the incubator at 37°C and 5%  $\text{CO}_2$  for 9 days, the medium was exchanged every 3 days. The samples were monitored using inverted optical microscope in a phase contrast mode.

[0050] TEM On day 9 of the culture the gels containing the cells were prefixed with 2% glutaraldehyde in culture medium at 4 °C for 1.5 hours. The samples then were washed in 0.1 M cacodilate buffer two times for 10 minutes and fixed in the Karnovsky fixative solution. Samples were pre-stained in 1%  $\text{OsO}_4$  in the cacodilate buffer and 2% of uranyl acetate in distilled water. Samples were dehydrated in ethanol gradient, and propylene oxide and embedded in Epon embedding resin. The ultrathin sections of the embedded samples were cut on the Leica ultracut. The sections were post-stained with lead citrate and uranyl acetate and examined the transmission electron microscope.

[0051] In both preparations, with and without addition of  $\text{Ca}^{+2}$ , gels were formed. The gel formed without addition of  $\text{Ca}^{+2}$  was weaker than one formed in the presence of  $\text{Ca}^{+2}$ . The ability of the culture medium to gel the negatively charged peptide-amphiphile solution may be due to the presence of the polyvalent metal ions in the culture medium. In both

preparations cells have survived during the 9 days period. Cells embedded in the gel were able to move and divide. The shape of the cells generally remained spherical. In contrast the same cells grown on 2-dimensional substrates tend to spread and form monolayers. It is suggested herein that any negatively charged peptide-amphiphile will gel in the presence of cell culture medium, regardless its actual sequence. Similar methods could be used by those skilled in the art to determine the movement and division of other cells in similar or different peptide amphiphile scaffolds.

**[0052]** While not wishing to be bound by theory, it would appear that the ability of the culture medium to gel the peptide-amphiphile solution is due to the presence of the polyvalent ions which may include but are not limited to metal ions, organic ions or complex ions.

**[0053]** The fact that the peptide amphiphile gels in the culture medium supports that gelation or self assembly of such peptide amphiphiles would also occur in the presence of body fluids, such as, for example, blood since they have similar inorganic chemical compositions. Therefore, the peptide-amphiphiles can be used in-situ applications, for example, for sealing blood vessels during surgical operations. At concentrations ranging from about 0.1% to about 15% by weight of peptide amphiphiles, and/or at concentrations above about 2.5 mg/ml, the self-assembly of peptide-amphiphiles occurs at physiological pH.

**[0054]** Advantages of the present invention include the ability to combine many types of cells with the scaffold precursors, to provide enzymes to naturally degrade the scaffold, and the ability to prepare scaffolds under physiological conditions.

**[0055]** All of the embodiments disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to

the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

**[0056]** While the principles of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions are added only by way of example and are not intended to limit, in any way, the scope of this invention. For instance, various peptide amphiphiles have been described in conjunction with specific residues and corresponding cell adhesion, but other residues can be used herewith to promote a particular cell adhesion and tissue growth on the nanostructures prepared therefrom. Likewise, while the present invention has been described as applicable to biometric material or tissue engineering, it is also contemplated that gels or related systems of such peptide amphiphiles can be used as a delivery platform or carrier for drugs, cells or other cellular or therapeutic material incorporated therein. Other advantages and features will become apparent from the claims filed hereafter, with the scope of such claims to be determined by their reasonable equivalents, as would be understood by those skilled in the art.

**[0057]** Although the present invention has been described in considerable detail with reference to certain preferred embodiments thereof, other versions are possible. Therefore the spirit and scope of the appended claims should not be limited to the description and the preferred versions contain within this specification.

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## CLAIMS

What is claimed:

- 1.. A self assembled peptide amphiphile gel comprising:

a gel comprised of a network one or more cylindrical fibrils, said fibrils comprised of self assembled peptide amphiphiles, said peptide amphiphiles having a hydrophobic tail portion and a peptide sequence portion attached to an end of the hydrophobic portion, said peptide sequence portion comprising a flexible linker peptide region, connected at a first end to the hydrophobic tail portion and the second end of the flexible linker peptide region connected to an end of a hydrophilic head group peptide region; and

cells within the network of fibrils of said gel

- 2.. The self assembled peptide amphiphile gel of claim 1 further comprising a fluid within said network of fibrils including polyvalent ions.
3. The self assembled peptide amphiphile gel of claim 1 wherein said gel further comprises a biologically active material within said network of fibrils chosen from the group consisting of humoral factors, angiogenic factors, antibiotics, antiinflammatories, growth factors and combination of these.
4. The self assembled peptide amphiphile gel of claim 1 wherein said cells are chosen from the group consisting of myocytes, adipocytes, fibromyoblasts, ectodermal cell, muscle cells, osteoblast, chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.

5. The self assembled peptide amphiphile gel of claim 1, wherein said peptide amphiphile has a cross linking peptide region, a first end of said cross linking region bonded to an end of the hydrophobic tail portion of the peptide amphiphile, and a second end of the cross linking peptide region bonded to the end of the flexible region, said cross linking region comprised of an amino acid with a functional moiety capable of intermolecular covalent bond formation.
6. The cross linking peptide region of claim 5, wherein said amino acid is cysteine.
7. The composition of claim 1 wherein the hydrophilic head group region of said peptide amphiphile has a peptide sequence is chosen from the group consisting of (SEQ ID NO:5-7) and combinations of these.
8. A composition comprising:
  - cylindrical fibrils, said cylindrical fibrils comprised of self assembled peptide amphiphiles, said peptide amphiphiles having a hydrophobic tail portion and a peptide sequence portion attached to an end of the hydrophobic portion, said peptide sequence portion comprising a flexible linker peptide region connected at a first end to the hydrophobic tail portion and the second end of the flexible linker peptide region connected to an end of a hydrophilic head group peptide; and
  - cells.
9. The composition of claim 8 further comprising polyvalent ions.
10. The composition of claim 8 further comprising a biologically active material chosen from the group consisting of humoral factors, angiogenic factors, antibiotics, antiinflammatories, growth factors and combination of these.
11. The composition of claim 8 wherein said cells are chosen from the group consisting of myocytes, adipocytes, fibrocytes, ectodermal cell, muscle cells, osteoblast,



- chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.
12. The composition of claim 1, wherein said peptide amphiphile has a cross linking peptide region, a first end of said cross linking region bonded to an end of the hydrophobic tail portion of the peptide amphiphile, and a second end of the cross linking peptide region bonded to the end of the flexible region, said cross linking region comprised of an amino acid with a functional moiety capable of intermolecular covalent bond formation.
  13. The cross linking peptide region of claim 13, wherein said amino acid is cysteine.
  14. The composition of claim 8 wherein the hydrophilic head group region of said peptide amphiphile has peptide sequence chosen from the group consisting of (SEQ ID NO:5-7) and combinations of these.
  15. A composition comprising: cells and peptide amphiphile, said peptide amphiphiles having a hydrophobic tail portion and a peptide sequence portion attached to an end of the hydrophobic portion, said peptide sequence portion comprising a flexible linker region connected at a first end to the hydrophobic tail portion and the second end of the flexible linker region connected to an end of a hydrophilic head group peptide, said cells and peptide-amphiphiles are in solution, said composition capable of forming a gel upon exposure to physiological conditions.
  16. The composition of claim 15 further comprising polyvalent ions.
  17. The composition of claim 15 further comprising a biologically active material chosen from the group consisting of humoral factors, angiogenic factors, antibiotics, antiinflammatories, growth factors and combination of these.
  18. The composition of claim 15 wherein the cells are selected from the group consisting of pluripotent cells, stem cells, precursor cells and combinations thereof.

19. The composition of claim 15 wherein the cells are selected from the group consisting of myocytes, adipocytes, fibromyoblasts, ectodermal cell, muscle cells, osteoblast, chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.
20. The self assembled peptide amphiphile gel of claim 15, wherein said peptide amphiphile has a cross linking peptide region, a first end of said cross linking region bonded to an end of the hydrophobic tail portion of the peptide amphiphile, and a second end of the cross linking peptide region bonded to the end of the flexible region, said cross linking region comprised of an amino acid with a functional moiety capable of intermolecular covalent bond formation.
21. The cross linking peptide region of claim 20, wherein said amino acid is cysteine.
22. The composition of claim 15 wherein the hydrophilic head group region of said peptide amphiphile has peptide sequence chosen from the group consisting of (SEQ ID NO:5-7) and combinations of these.
23. A composition comprising: a solution of a peptide-amphiphile, cells; and a reagent to induce gelation of said peptide amphiphile; said peptide amphiphiles having a hydrophobic tail portion and a peptide sequence portion attached to an end of the hydrophobic portion, said peptide sequence portion comprising a flexible linker peptide region connected at a first end to the hydrophobic tail portion and the second end of the flexible linker peptide region connected to an end of a hydrophilic head group peptide.
24. The composition of claim 23 further comprising a biologically active material chosen from the group consisting of humoral factors, angiogenic factors, antibiotics, antiinflammatories, growth factors and combination of these.
25. The composition of claim 23 wherein the cells are selected from the group consisting of pluripotent cells, stem cells, precursor cells and combinations thereof.

26. The composition of claim 23 wherein the cells are selected from the group consisting of myocytes, adipocytes, fibromyoblasts, ectodermal cell, muscle cells, osteoblast, chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.
27. The self assembled peptide amphiphile gel of claim 23, wherein said peptide amphiphile has a cross linking peptide region, a first end of said cross linking region bonded to an end of the hydrophobic tail portion of the peptide amphiphile, and a second end of the cross linking peptide region bonded to the end of the flexible region, said cross linking region comprised of an amino acid with a functional moiety capable of intermolecular covalent bond formation..
28. The cross linking peptide region of claim 27, wherein said amino acid is cysteine.
29. The composition of claim 23 wherein the hydrophilic head group region of said peptide amphiphile has peptide sequence chosen from the group consisting of (SEQ ID NO:5-7) and combinations of these.
30. A scaffold for culturing cells comprising;  
a network of cylindrical fibrils comprised of self assembled peptide amphiphiles; and cells attached to said network, wherein said fibrils are formed by self assemble of peptide amphiphiles mixed in a solution with polyvalent cations, said peptide amphiphile comprising a hydrophobic tail portion and a peptide sequence portion attached to the hydrophobic portion, said peptide sequence portion comprising a flexible linker peptide region connected at a first end to the hydrophobic tail portion and the second end of the flexible linker peptide region connected to an end of a hydrophilic head group peptide region.
31. A method of growing cell in an animal comprising: injecting the composition of any of claims 8, 15, 23, or 30 into said animal.

32. A method of growing cell in an animal comprising:
- forming an implantable substrate that is a gel comprised of a network of cylindrical fibrils of self assembled peptide amphiphiles with the composition of any of claims 8, 15, 23 or 30; and
  - implanting said substrate into the animal.
33. A method for making a gel comprised of a network of cylindrical fibrils of self assembled peptide amphiphiles for culturing cells and tissue comprising:
- combining a solution comprising peptide amphiphiles with a solution comprising cells to form a mixture that is capable of self assembly into a peptide amphiphile gel having said cells within said gel.
34. The method of claim 33 further comprising the step of adding multivalent ions to said mixture.
35. The method of claim 33 wherein said solution comprising cells is a cell culture medium having polyvalent ions.
36. The method of claim 33 wherein said gel is comprised of peptide amphiphiles having a biological signal for promoting the interaction of said cells with said gel.
37. A method for forming a tissue within an animal comprising:
- mixing a solution of a peptide-amphiphile composition with dissociated cells to form a mixture; and
  - placing the mixture into the animal to thereby form a self-assembled peptide-amphiphile nanofiber network having cells dispersed therein inside the animal.
38. The method of claim 37 wherein the act of placing the mixture into the animal is chosen from the group consisting of injection and implantation.
39. The method of claim 37 wherein gelation of the mixture comprising cells and peptide amphiphiles is initiated prior to placing the mixture in the animal.

40. The method of claim 37 wherein gelation of the mixture comprising cells and peptide amphiphiles is initiated after placing the mixture into the animal.
41. The method of claim 40 wherein the gelation of the peptide-amphiphile solution occurs by using ions, altering the pH or changing the temperature.
42. The method of claim 37 wherein the cells are selected from the group consisting of chondrocytes, muscle cells, fibroblasts, and cells acting primarily to synthesize, secrete or metabolize materials.
43. The method of claim 37 wherein the cells are selected from the group consisting of pluripotent cells, stem cells, precursor cells and combinations thereof.
44. The method of claim 37 wherein the cells are selected from the group consisting of myocytes, adipocytes, fibrocytes, ectodermal cell, muscle cells, osteoblast, chondrocyte, endothelial cells, fibroblast, pancreatic cells, hepatocyte, bile duct cells, bone marrow cells, neural cells, genitourinary cells and combinations thereof.
45. A kit for formation of a gel including cells at a site in an animal comprising an injectable solution comprising a cells and peptide amphiphiles, said solution in combination with means for injecting the cell/peptide-amphiphile solution into the site in the patient where gel is to be formed.

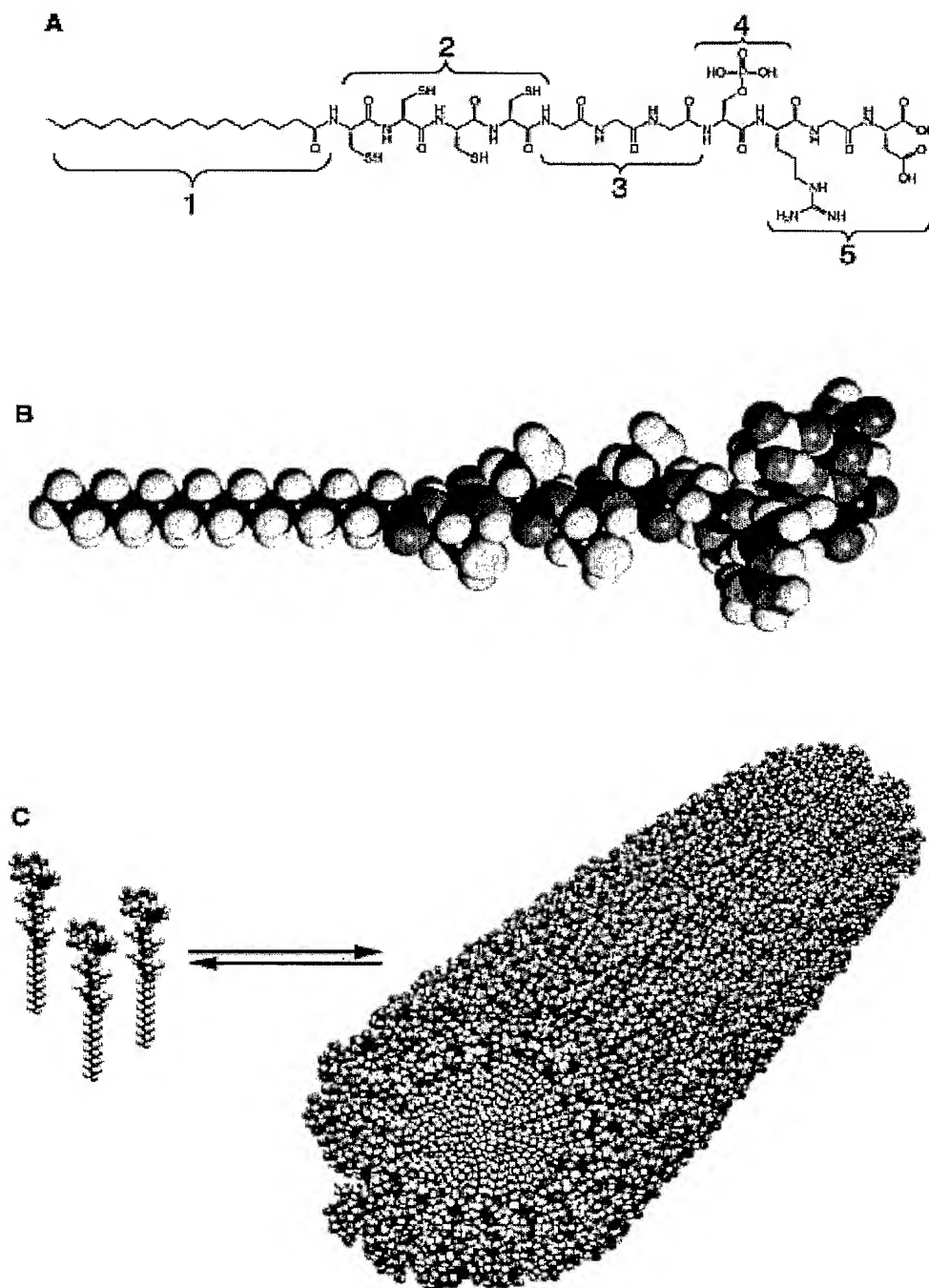
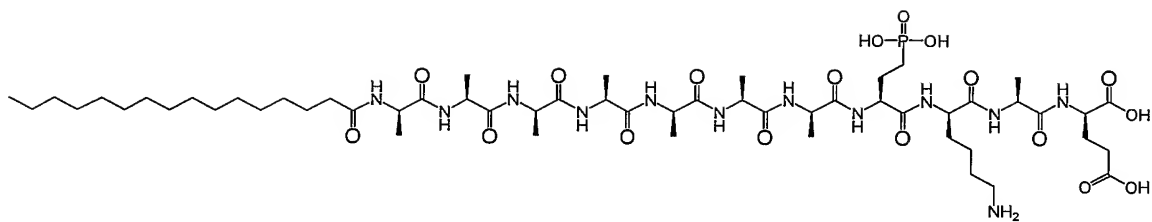
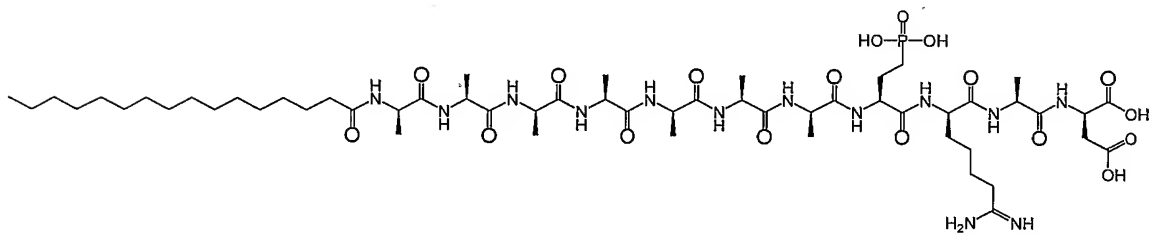


FIGURE 1

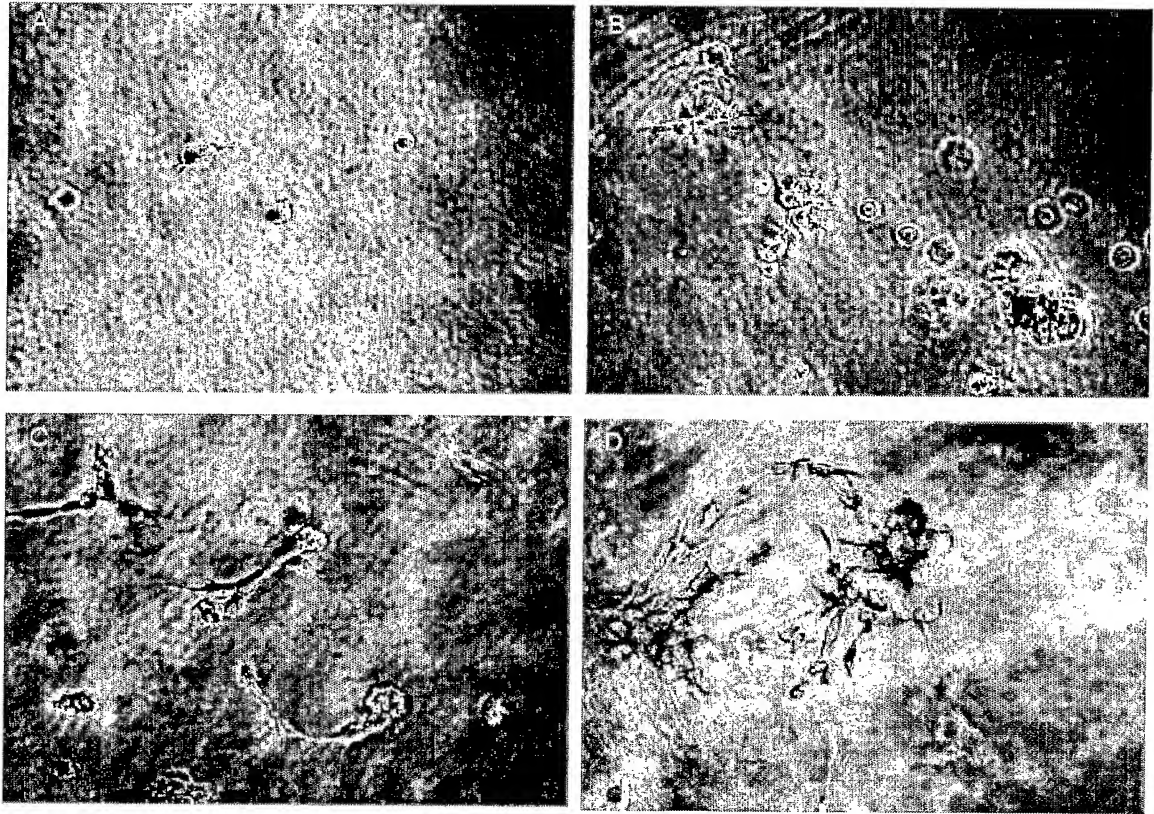


MOLECULE 1



MOLECULE 2

**FIGURE 2**



**FIGURE 3**



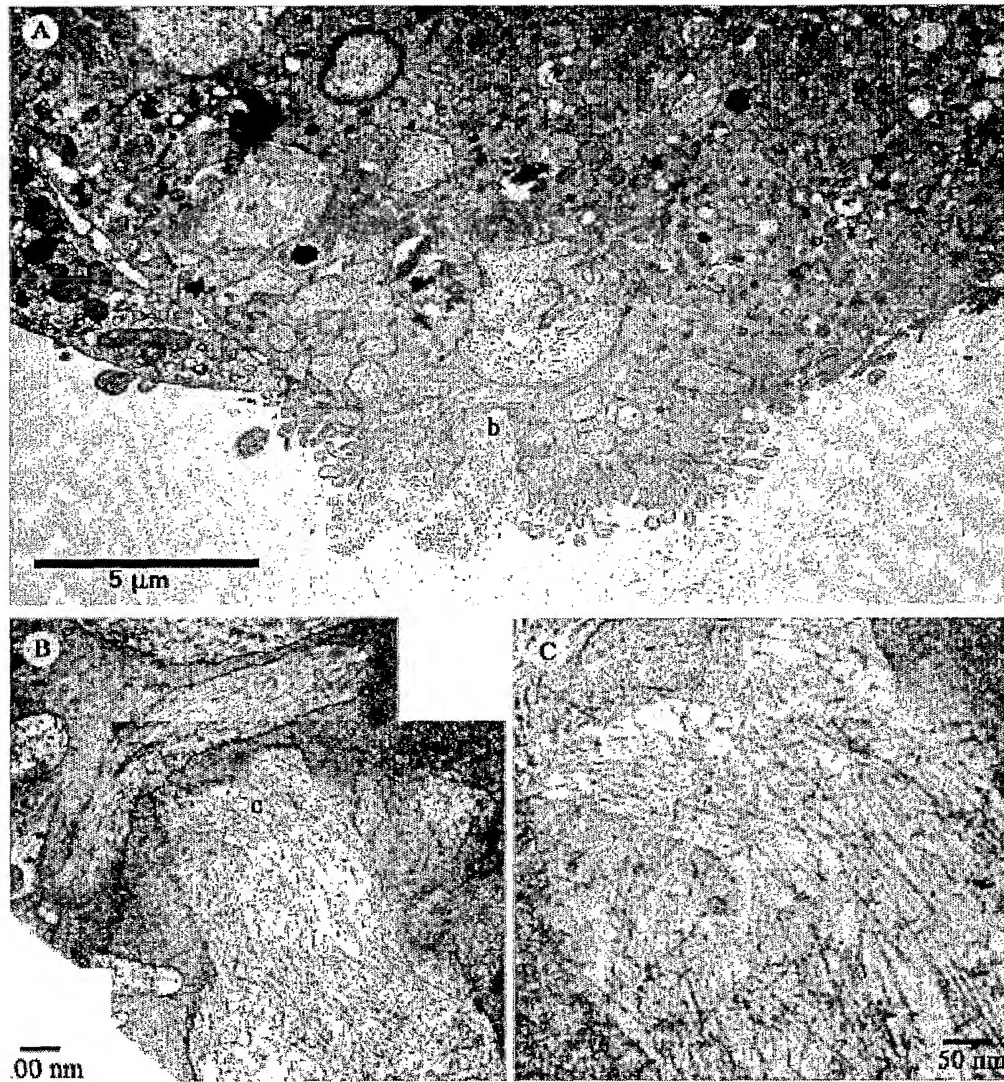


FIGURE 4

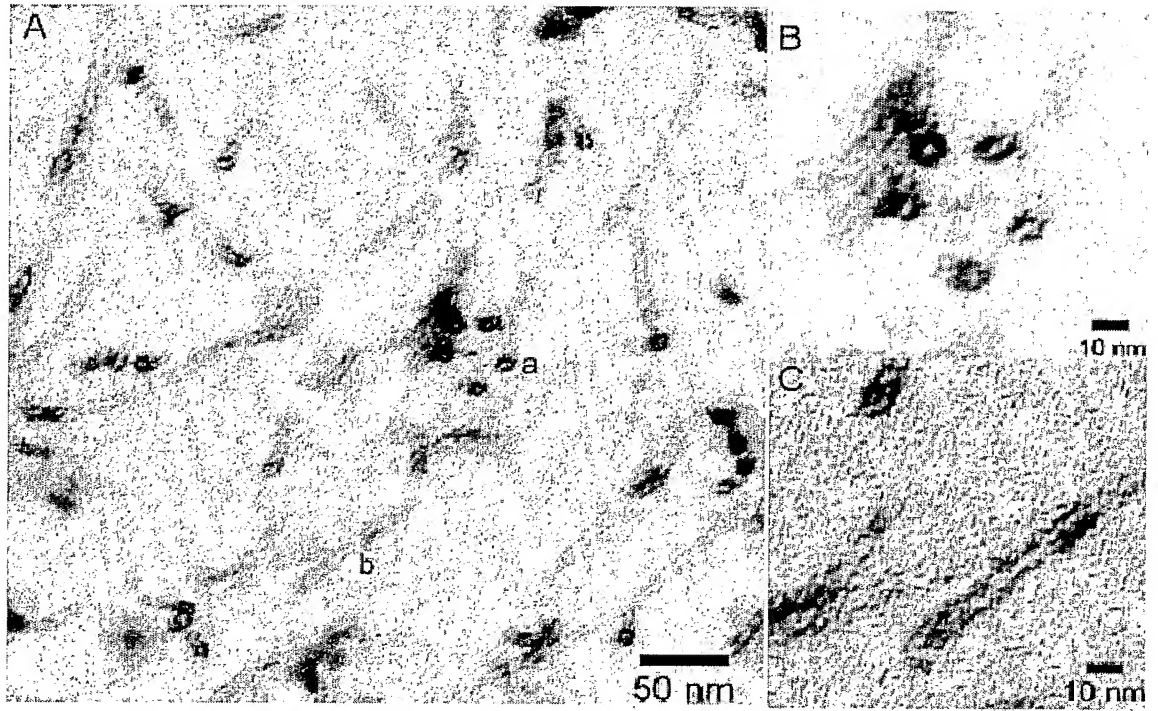


FIGURE 5

## SEQUENCE LISTING

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<120> PEPTIDE AMPHIPHILE SOLUTIONS & SELF-ASSEMBLED PEPTIDE  
NANOFIBER NETWORKS

<130> Attorney Docket Number 1234343434

<141> April 2, 2003

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